Canine conceptus-maternal communication during maintenance and termination of pregnancy, including the role of species-specific decidualization

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Abstract: Among domestic animal species, the reproductive biology of the dog belongs to the most peculiar. This includes the conceptus-maternal communication and endocrine mechanisms involved in maintenance of pregnancy. Dogs fully depend on luteal progesterone (P4) throughout pregnancy, with similar steroid secretion patterns in pregnant and non-pregnant bitches until prepartum luteolysis. Thus, dogs lack the classical recognition of pregnancy. The luteal P4 is the most important hormone regulating the onset and maintenance of pregnancy in previously estrogenized bitches. Although the canine uterus is exposed to high P4 levels, decidualization is not spontaneous but induced by the presence of embryos. Following implantation, decidualization continues, associated with development of the invasive endotheliochorial placenta, leading to establishment of maternal decidual cells expressing the nuclear P4 receptor (PGR). Consequently, although not producing steroids, the canine placenta remains highly sensitive to circulating ovarian steroids. The placental conceptus-maternal communication is responsible for the maintenance of pregnancy, with functional withdrawal of PGR evoking a luteolytic cascade with prepartum PGF2 release. The fetal trophoblast is the major source of prepartum placental prostaglandins. This conceptus-maternal communication is unique to the dog and has clinical implications. Due to luteal steroids, there is no prepartum estradiol increase. Elevated cortisol levels are observed irregularly. This emphasizes the unique character of canine reproductive physiology and the challenges in transferring translational research to the dog. Further research is needed for better understanding of canine reproduction and improving clinical protocols, including the latest results obtained from applying modern laboratory technologies such as the transcriptomic approach.

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ABSTRACT

Among domestic animal species, the reproductive biology of the dog belongs to the most peculiar. This includes the conceptus-maternal communication and endocrine mechanisms involved in maintenance of pregnancy. Dogs fully depend on luteal progesterone (P4) throughout pregnancy, with similar steroid secretion patterns in pregnant and non-pregnant bitches until prepartum luteolysis. Thus, dogs lack the classical recognition of pregnancy. The luteal P4 is the most important hormone regulating the onset and maintenance of pregnancy in previously estrogenized bitches. Although the canine uterus is exposed to high P4 levels, decidualization is not spontaneous but induced by the presence of embryos. Following implantation, decidualization continues, associated with development of the invasive endotheliochorial placenta, leading to establishment of maternal decidual cells expressing the nuclear P4 receptor (PGR). Consequently, although not producing steroids, the canine placenta remains highly sensitive to circulating ovarian steroids. The placental conceptus-maternal communication is responsible for the maintenance of pregnancy, with functional withdrawal of PGR evoking a luteolytic cascade with prepartum PGF2α release. The fetal trophoblast is the major source of prepartum placental prostaglandins. This conceptus-maternal communication is unique to the dog and has clinical implications. Due to luteal steroids, there is no prepartum estradiol increase. Elevated cortisol levels are observed irregularly. This emphasizes the unique character of canine reproductive physiology and the challenges in transferring translational research to the dog. Further research is needed for better understanding of canine reproduction and improving clinical protocols, including the latest results obtained from applying modern laboratory technologies such as the transcriptomic approach.

1. Introduction

Because it is characterized by species-specific regulatory mechanisms, the dog appears to be an interesting model for investigating comparative and evolutionarily-determined aspects of reproduction in mammals. The OMNIA (Online Mendelian Inheritance in Animals; http://omia.angis.org.au/home/) database lists the dog as the domestic animal species with the highest number of traits/disorders serving as potential models for human diseases. Consequently, together with the social and economic value of the dog as one of the most important pets, investigations into clinical and basic research related aspects of canine reproduction attract scientific interest.

Whereas earlier studies were focused predominantly on endocrine events regulating canine reproductive function, by using modern laboratory methods of molecular and cell biology and applying them to the canine species, considerable progress has been achieved recently in understanding the reproductive physiology of a dog. This predominantly addresses ovarian/luteal, uterine and placental functions. Yet, many questions are waiting for answers, including those related to mechanisms involved in establishment, maintenance and termination of canine pregnancy.

Pointing towards the definition of conceptus as the embryo/fetus including all its associated membranes, in the present manuscript, the term *conceptus-maternal communication* is used to describe both the embryo-maternal and feto-maternal communication. With relation to the placental communication between the

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two entities (i.e. the mother and the fetus), the term placental feto-maternal communication will apply.

Accordingly, adding to comprehensive reviews, including some recent contributions from our group, and those of other researchers, regarding regulation of canine ovarian function and the conceptus-maternal communication (e.g. Refs. [1–8]), here, an overview of some well-established facts as well as more recent aspects and views on canine reproduction are discussed. Included are the newly established and available cell culture model of canine decidualization [9,10] and insights from the results obtained by applying modern technologies, like the transcriptomic approach [11,12].

The focus of the present work is on the endocrine milieu and morpho-functional aspects of the embryo- and feto-maternal communication during implantation, placentation and termination of pregnancy in the dog. Highlighted are the importance of the decidualization process, and of the maternally-derived decidual cells arising from this process, in the underlying regulatory mechanisms, as well as their possible clinical and translational implications.

1.1. Recalling some well-known facts

The domestic dog is generally classified as an aseasonal, monoestrous breeder. This means that the estrous cycle can occur at any time throughout the year and ovarian cycles are separated from each other by an obligatory quiescence phase, referred to as anestrus. Also, the dog is a spontaneous ovulator. Thus, each “heat” phase is followed by ovulation, ending in the establishment of a long-lasting luteal phase (diestrus).

At this point, it seems important to mention that ovulation in the dog is triggered by concomitantly decreasing estrogen levels and increasing progesterone (P4) concentrations (thereby lowering the E2:P4 ratio) (reviewed in Refs. [2,7,13]). The latter, i.e., rising P4 concentrations, are derived from preovulatory follicular luteinization which is exceptionally strong in this species (s. reviewed in Refs. [5,6]).

The most peculiar features underlying the species-specific characteristics of canine reproductive physiology and providing the proper endocrine and functional context for better understanding of the conceptus-maternal interaction in the dog, include the following facts:

i) The *corpus luteum* (CL) serves as the sole source of P4, both in non-pregnant animals and during pregnancy; this is due to the lack of placental steroid synthesis [14,15]. In fact, the dog is the only domestic animal species devoid of placental steroidogenesis. This also positions the CL as a central organ responsible for the successful outcome of pregnancy in the dog.

   In more detail, there is no P450scc activity in the canine placenta and no hints could be obtained of placental-specific steroid synthesis associated with the expression of 3bHSD or P450scc [14,15]. Such activities would be needed to define this organ as being steroidogenic. A further clue for the lack of placental steroid synthesis in the dog is the concomitant decrease of peripheral P4 and E2 levels observed during prepartum luteolysis, indicating the CL source of these steroids [14,16,17]. This has been further substantiated in our recent findings with the transcriptomic approach in which no placental steroidogenic activity was determined, but rather a response to the functional withdrawal of steroid was noted in the CL during prepartum luteolysis [18].

ii) Similar to other carnivores, in dogs there is invasive endotheliochorial placentation. As a component of species-specific decidualization, maternal stroma-derived decidual cells develop (presented in a greater detail elsewhere). By responding to circulating steroids, predominantly P4, and communicating with fetal trophoblast, these cells become an important partner in the conceptus-maternal dialog ensuring the maintenance of pregnancy [7,19–21]. Disturbances in this conceptus-maternal interaction will unequivocally lead to parturition/abortion.

iii) Lacking a prepartum E2 increase, the luteolytic cascade in pregnant dogs at the end of gestation diverges from that in other domestic animal species. In this context, regarding the prepartum luteolysis, the situation observed in pregnant bitches differs from that observed in the absence of pregnancy where no luteolytic principle is observed, either of uterine or of luteal origin (reviewed in Refs. [5,6]). Moreover, similar to cats and humans, at least in non-pregnant dogs there is no uterine luteolysin, as hysterectomy does not affect ovarian function and is followed by normal cyclicity [22,23]. Consequently, the lack of an active luteolytic principle, and its resulting extended pseudopregnancy in non-pregnant dogs, strongly contrasts with the prepartum release of utero-placental PGF2α [5,11,19,21,24,25]. The latter appears to be involved in both luteolysis and uterine myocontractile activity [18,19,21,26].

iv) In contrast to livestock, in dogs the peri-partum peripheral increase in cortisol is irregularly observed, displaying high individual variations. It appears also to be more strongly linked to parturition itself [24,25,27] than to the prepartum luteolysis. Notably, however, as indicated previously [11,27,28], the peripheral concentrations of cortisol may not reflect its local activities.

v) Since P4 and E2 show similar profiles in pregnant and non-pregnant cycles, and due to the exaggeratedly high PRL levels in overtly pseudopregnant bitches (*lactatio falsa*), PRL cannot be used as a marker of pregnancy, even if its concentrations increase during the second half of pregnancy. Thus, following implantation and placentation, placental RLN remains so far the only available and reliable marker of canine pregnancy from about day 25–30 [29,30].

Some of the above-mentioned endocrinological and regulatory events will be elaborated in more detail below. Collectively, however, it is apparent that even while following the same goal of maximal reproductive success, in the dog different regulatory strategies apply than in other domestic animal species. This is particularly visible at both ends of canine pregnancy, i.e., during its establishment and at the prepartum luteolytic cascade.

1.2. Maternal recognition of pregnancy

Unlike in virtually all other mammals, final maturation of canine oocytes takes place within the oviduct during 2–3 days after ovulation (i.e., 4–5 days following the pre-ovulatory LH surge), before they reach the status of secondary oocytes and can be fertilized [31]. Embryos may arrive relatively late in the uterus, i.e., 7–10 days following fertilization [32,33], while their intrauterine migration can take up to 9–10 days [34,35]. However, apposition of blastocysts to the uterine epithelium can already be observed as early as days 12–14 after fertilization [36,37]. Implantation and invasion are synchronized and occur from days 17–18 of embryonal life [9,36,37].

The lack of luteolysis in non-pregnant dogs precludes use of the classical definition of maternal recognition of pregnancy identified

as an anti-luteolytic measure securing the maintenance of luteal P4 production and facilitating embryo implantation [8,38]. In contrast, in both pregnant and non-pregnant dogs the uterus is exposed to high P4 concentrations, exceeding those needed for implantation and maintenance of pregnancy, without, however, inducing the spontaneous morphological decidualization observed in humans [9,39]. Only following implantation does the embryo-maternal contact become more intimate and then morphological changes can be observed in the uterus [9,40]. Accordingly, recently a new definition has been proposed that more accurately reflects the species-specific regulatory mechanisms in dogs, and which describes maternal recognition as a morphological and functional relationship between the uterus, the embryo and the CL as the sole source of P4 in the dog [8].

A more detailed overview of the early developmental events associated with the establishment of pregnancy in the dog has been presented recently [8].

2. Endocrinology of canine pregnancy

2.1. The luteal source of progesterone

In dogs, as in other mammalian species, a constant supply of P4 is crucial for the onset and maintenance of pregnancy. The use of P4 signaling-disruptors (e.g., aglepristone, mifepristone) for the induction of preterm luteolysis and/or abortion highlights the pivotal role of P4 as a luteotropic factor that is vital for the maintenance of canine pregnancy [21,41,42].

Thus, a clear understanding of CL regulation and its physiology is of outmost importance for the study of canine pregnancy and thus needs to be addressed.

Across mammals, the life cycle of a CL is classically described as starting immediately after ovulation, with differentiation of the remaining follicular granulosa and theca cells into small and large luteal cells. This description does not, however, fully reflect the situation observed in the dog. Thus, a slow rise in P4 circulatory levels can already be observed during proestrus, reflecting the presence of a pre-ovulatory luteinization [43,44]. Indeed, this species-specific phenomenon was described for the first time by von Bischoff in his pioneering work from 1845 [45]. Later, at the time of the LH surge, which determines the transition from proestrus to estrus, P4 levels start to rise rapidly, with falling E2 levels, and ovulation takes place 1–3 days after the LH surge under high levels of circulating P4 (>5 ng/ml) [46,47]. At this time, strong luteinization of cells from the granulosa and theca layers can be observed [48]. It is worth mentioning that, even though both follicular layers appear to contribute to luteal formation, morphologically and functionally only one type of luteal cell can be observed in the canine CL, contrasting with other domestic animals ([reviewed in Refs. [49,50]). After ovulation, the CL starts to develop rapidly. This is reflected in the quick rise of circulating P4 levels, which reach their highest values between days 15 and 30 post-ovulation, averaging from 30 to 35 ng/ml [46,51]. However, large breed and individual variations can be observed, with some animals attaining P4 levels as high as 90 ng/ml [52]. Consequently, during implantation and initiation of placentation, as mentioned elsewhere, the reproductive tract is exposed to high P4 concentrations. The E2 profiles roughly follow those of P4 and even at mid-gestation/diestrus never exceed the maximum levels observed during proestrus; no pregnancy-specific increase in E2 is observed (reviewed in Refs. [7,8,13]). Interestingly, as shown in experiments with naturally estrogenized bitches that were ovariectomized at day 14 after the LH surge, exogenous supplementation with P4 alone as the main steroid is sufficient for the initiation and maintenance of gestation ([2,53] and reviewed in Ref. [8]).

Circulating levels of P4 start to decrease slowly at mid-diestrus, marking the shift between a developed CL with a high steroidogenic output into a slowly regressing CL. The first signs of morphological degeneration, as reflected in further decreasing steroidogenic output, can be observed shortly thereafter, at approximately day 35 after ovulation [3,54]. The PGF2α-receptor (FP) is constitutively expressed in the CL of both pregnant and non-pregnant dogs, with expression levels increasing during diestrus, even if the intraluteal content of PGF2α is low, mirrored by strongly supressed expression levels of the respective synthases (PTGFS, PTGS2) [19,55–57]. Indeed, despite the lack of an endogenous luteolysin in non-pregnant dogs, the canine CL remains sensitive to exogenously applied PGF2α, even as early as at day 5 of cytologic diestrus [58–60]. The side effects observed following this treatment, such as emesis, diarrhea and panting, indicate the usage of PGF2α to be unphysiological [59]. On the other hand, these side effects may be associated with the response to the variably high dosages of exogenously used PGF2α. Consequently, CL regression appears to be an inherently regulated process of functional degeneration, with a prolonged duration, which in non-pregnant animals frequently exceeds the length of a pregnancy [49]. In this, the dog is the only domestic animal species in which an inverse relationship may occur between the length of pregnancy and pseudopregnancy (non-pregnant cycle).

The slow luteal regression/degeneration is accompanied by a continuous decrease in P4 levels until they fall below 1 ng/ml, marking (per definition) the onset of anestrus [46,47,49]. In pregnant animals, however, this slow P4 decrease is abruptly interrupted around day 60 of the CL life span, with the induction of prepartum luteolysis and a steep decrease in P4 levels. This active termination of CL function is associated with increased production of utero-placental PGF2α [21,61–64]. It is noteworthy that high individual and diurnal variations in circulating levels of P4 are observed [65].

More detailed descriptions of the stages of CL development in the dog can be found under [5,6,48].

2.2. Maintenance of CL function and provision of progesterone for successful pregnancy

The regulation of CL maintenance and function is also quite peculiar in the domestic dog. In early diestric bitches (day 4 after ovulation), ablation of the hypophysis had only temporary effects on the circulating levels of P4 which returned to normal levels by 6–10 days after surgery [66]. Thus, a transitional independence from hypophyseal support was postulated within the first 3–4 weeks (24–28 days) of the luteal phase, because in bitches hypophysectomized at day 18 after ovulation the P4 levels did not recover [67]. The high expression of prostaglandin synthase 2 (PTGS2/COX2) within this period, as well as of prostaglandin E2 (PGE2) synthase (PTGES) and the two PGE2 receptors 2 and 4 (EP2/PTGER2 and EP4/PTGER4), suggests an auto/paracrine role of prostaglandins (PGs) in CL regulation [57,68]. In addition, PGE2 can modulate steroidogenesis as well as expression of endothelin receptor B (a potent vasodilator) and of PRL receptor (PRLR) in luteal cells *in vitro* [48,69,70]. Finally, *in vivo* inhibition of COX2 and the consequent decrease in intra-luteal prostaglandins further affected not only the steroidogenic capacity of the CL but also its sensitivity to PRL, vascularization and the immune system [48,71,72]. These findings place PGs among the main luteotropic factors in early canine diestrus. Nevertheless, support from gonadotropins is required for the maintenance of CL function in the second half of diestrus/pregnancy, with both PRL and LH having luteotropic roles [66,73–75]. However, PRL and not LH becomes an absolutely required luteotropic factor from approximately day 25 after

ovulation onward [67,73,76,77]. The disruption of PRL function (with bromocriptine, a dopamine receptor agonist) severely affects CL function, leading to premature luteolysis and inducing abortion [73,75]. These effects can be reversed with the administration of PRL but not LH [73]. Prolactin levels rise towards mid-diestrus and remain high during the remainder of diestrus, despite individual and diurnal variations [44,73,78,79]. The role of PRL appears to be more supportive, as even under high levels of this luteotropin, luteal regression still takes place. This could be associated with decreasing PGE2 support and its stimulatory role on PRLR expression, resulting in desensitization of the CL towards PRL [48,80]. The prepartum drop in P4 is needed for parturition to occur because of the signaling function of P4/PGR during the luteolytic cascade [20,21], and is associated with the prepartum PGF2α release. As mentioned elsewhere, and adding to the hormonal milieu during pregnancy, cortisol appears to be less prominent during canine pregnancy. In fact, increased circulating levels of cortisol, most probably derived from the fetal adrenals, have been described as erratic in the bitch and, thus, not mandatory for normal parturition [62,81]. Nevertheless, utero-placental expression of glucocorticoid receptor is elevated at the time of normal prepartum luteolysis [28] (elaborated further elsewhere).

2.3. Markers of pregnancy

Markers of pregnancy are useful in veterinary medicine for the early detection of pregnancy. In the dog, however, identification of such markers is quite challenging. The detection of pregnancy based on the presence/absence of luteal hormones (P4 or E2) or PRL is unreliable in the dog (see above). Nevertheless, the presence of free-floating embryos modulates uterine expression of different factors like PRLR, insulin-like growth factors (IGFs), members of the prostaglandin family (e.g. PTGES, PGT), the VEGF system, extracellular matrix (ECM) components and immune-related factors, as also found in our experiments and being further underpinned by the latest data collected from a transcriptomic approach [12,39,82,83]. In one of the latter studies, involving genome-wide analysis of gene expression [12], we found over 400 genes that were highly modulated by the presence of early pre-implantation embryos at day 10–12. These genes may yield potential early pregnancy markers. Interestingly, our studies [9,12,39,40] indicate that the first uterine conceptus-maternal contact in the dog primarily involves biochemical and not morphological modifications of the uterus.

In this respect, regarding early embryo-maternal contacts, the most important findings from our studies were the effects exerted by free-floating embryos on uterine matrix assembly and biochemical modulation of uterine extracellular matrix (ECM) (e.g., of ECM1, TIMP2, TIMP4 or LAMA2). The expression of major collagens (COL1, -3 and -4) was, however, not affected by free-floating embryos [39,40]. The effects exerted by pre-attachment embryos on ECM were followed by over-represented genes and functional terms indicating immunomodulatory and/or inflammatory responses [12]. This was underlined by the associated prevailing functional networks and pathways indicating acute phase response signaling, activation of the complement system and pathways associated with diapedesis and adhesion of immune cells [12]. These two functional features, i.e., modulation of ECM and strong immunomodulation, show the nature of the early conceptus-maternal communication in the dog initiated by free-floating embryos. It also appears plausible that functional intersections between these two entities play a role in the regulation of decidualization, implantation and placentation, and in modulating uterine immune function to prevent the rejection of embryos. They also indicate the focus of ongoing and future investigations both in clinical and basic science settings.

In other words, it becomes apparent that, despite lacking an anti-luteolytic signal and without strongly modifying the morphological appearance of the uterus, there is a range of factors involved in early embryo-maternal signaling already at the pre-implantation stage of pregnancy, predominantly linked to secretory and adhesive activities of the uterus, associated with preparation for implantation and trophoblast invasion, rather than with proliferative activity of uterine structures [12,40].

Another interesting insight from our study applying the transcriptomic approach [12] is the good match in terms of commonly expressed genes between the early pregnant canine uterus exposed to free-floating embryos and the human uterus during the window of implantation (6–10 days after ovulation). This was attributed to P4-mediated effects and the ongoing decidualization [12].

The species-specific regulatory mechanisms during canine recognition of pregnancy were highlighted by over 1900 genes exclusively expressed in the canine uterus when compared with other domestic animal species (cattle, pig, horse) and humans. Most of these genes were linked to mitochondrial function, and to regulation of genomic and transcriptional activity [12].

With regard to the protection of implanting embryos, our recent as yet unpublished results indicate a shift from pro- to anti-inflammatory events during implantation (day 17), implied, for example, by increased presence of markers of Treg lymphocytes and decreased levels of MHCII or CD4.

Some of the immune factors potentially modulated by early embryo-maternal contact were investigated earlier, such as acute phase proteins (APPs: e.g., serum fibrinogen, glycoprotein, α2 globulin, C-reactive protein, and serum amyloid A). These showed increased circulating levels in early pregnant bitches and their potential use as pregnancy markers has been assessed in different studies [84–87]. Some more recent approaches included assessment of expression of some other immune-system related factors in early pregnant, preimplantation canine uteri, as well as selected heat shock proteins (e.g., HSP60 or HSP70) in the serum of early pregnant bitches [88–91]. Nevertheless, considering the role of many of these factors as first responders in inflammation, their strongly varying levels may be affected by the health status of the bitch, thereby so far limiting their utility in the diagnosis of pregnancy.

Consequently, until now, the only reliable marker of pregnancy identified in the dog is the hormonal polypeptide relaxin (RLN). This hormone is classically known for its remodeling effects in the pubic ligament and facilitating the passage of fetuses through the birth canal during whelping [92,93]. RLN becomes detectable in canine maternal blood around days 20–25 of pregnancy, i.e., after implantation and placentation [65,94]. After this, circulating levels continue to increase (peaking 2–3 weeks before parturition) and remaining high until delivery [30,65,94]. After parturition, RLN levels usually decrease below detection limits within a few days. Differently from other species (e.g., human, rat, pig), this hormone is only detectable in pregnant dogs [65]. Recently, however, the presence of the RLN system was confirmed in the canine CL, with expression profiles implying its local luteotropic function [95]. By applying canine-specific antibodies the expression and localization of the RLN system was investigated in utero-placental compartments throughout pregnancy [96]. The highest RLN levels were found in fetal cytotrophoblast. Clearly detectable expression of RLN receptor 1 (RXFP1) in maternal endothelial cells and fetal trophoblast, and of RXFP2 in decidual cells, suggest possible auto/paracrine effects of RLN in the placenta [96]. Going beyond that, the expression of the RLN system was also confirmed in the canine adenohypophysis, in PRL-secreting cells [95]. Thus, besides its classical role in the preparation of the birth canal, in the dog, RLN

appears to have local regulatory effects in the utero-placental compartments. Additionally, via the endocrine route, it may be possibly involved in central regulation of PRL secretion, being responsible for the elevated levels of PRL detected in pregnant dogs [96]. Nevertheless, these effects still need further confirmation.

3. Decidualization

3.1. General aspects

The crucial steps toward the establishment of pregnancy include well-timed and orchestrated communication between the embryo and maternal uterine structures.

Depending on how much cells from the outer layer of the blastocyst (trophoblasts) invade the uterine epithelium, contact between the conceptus and uterus becomes more intimate, and in species exhibiting invasive types of placentation, leads to strong morphological and biochemical reorganization of endometrial stromal compartments referred to as decidualization. This accounts for endotheliochorial and hemochorial types of placentation, in which strong erosion of the uterus is observed. Importantly, failure to decidualize results in loss of pregnancy [97].

In some primate species, like humans, decidualization is cyclically induced by P4, takes place after ovulation in every menstrual cycle [98] and is associated with strong remodeling of endometrial structures involving changes in the spiral arteries [99,100] and strong immunomodulation [101]. In the absence of implantation, concomitantly with falling circulating P4 levels, the endometrial lining is shed as decidua, giving the name to the process which derives from the latin term "*decidere*", meaning to "fall off". Contrasting with this is the process of decidualization in rodents (e.g., mice and rats) in which decidualization occurs only if conceptuses are present in the uterus and provide an implantation stimulus [102,103]. Notably, decidualization can be induced in rodents when an artificial stimulus is applied, however, signaling from embryos remains essential to fully evoke the para/autocrine pathways associated with decidua formation [102,103].

The term "decidua" has also been adopted to describe all placental types in mammals in which a more intimate, invasive placentation takes place and in which shedding of fetal membranes is not possible without expelling maternal uterine tissues. This type of placenta is referred to as "*placenta deciduata*" and describes the endotheliochorial placentae of carnivores, including dogs and cats, and hemochorial placentae of humans and rodents. In contrast, the epitheliochorial placenta is referred to as "*placenta adeciduata*".

Decidual cells are epithelioid-like cells derived from maternal stromal cells and undergo a mesenchymal-epithelial transition reflected in increased expression of basal lamina-associated COL4 and suppression of some stromal cell markers like CD10/NEP (neutral endopeptidase) [104]. As shown in the same study using an *in vitro* model [104], this process in human decidual cells is reversible upon hormonal withdrawal. These cells play a variety of roles, including nutritional support for the conceptus, regulation of trophoblast invasion, modulation of immune response and immunological protection of the fetus, as well as production of ECM, hormones and growth factors [98,105–108]. The canine species-specific features of decidual cells are presented in the respective chapter below.

3.2. Mechanisms of decidualization

Most of the knowledge about the decidualization process comes from investigations in humans and rodents. This information indicates that the major pathway involved in decidualization is the cAMP/PKA pathway [98,109,110]. Although P4 is one of the important players, it is not the only factor responsible for decidualization in humans [106,111], but initiates a cAMP-dependent signaling cascade [98]. Further, P4 is responsible for attraction of natural killer (NK) cells involved in promoting vascularization, production of growth factors and stimulating chemokine production [112,113], and is responsible for the so-called P4 block exerted upon the myometrium, ensuring its quiescence during the luteal phase and pregnancy [114]. The withdrawal of P4 function constitutes a part of the signaling cascade that results in menses and shedding of decidua in women, which are processes associated with induction of pro-inflammatory reactions [115]. This underlies the immunomodulatory and immunosuppressive properties of P4 exerted through decidual formation. Among factors induced by P4 during decidualization in humans and rodents are PRL, IGF1 and -2 and IGF-binding protein-1 (IGFB1), accounting for the most prominent so-called decidualization markers that are important for this process to occur [110,116–120].

For research purposes, decidualization can be induced in isolated uterine stromal cells by cAMP and other cAMP-inducing stimuli, such as PGE2. This also applies to the canine species, as shown in our investigations [9,10], discussed below in greater detail. In human decidual cells, PGE2 was shown to accelerate P4-dependent decidualization [121]. cAMP not only mediates the P4-induced decidualization, but is more efficient than P4 in activating expression of decidualization markers *in vitro* [110], implying interactions between different cellular, and presumably endocrine, components that are important for this process *in vivo*. Nevertheless, *in vitro* models are valuable tools allowing targeted studies into the underlying molecular biological mechanisms.

3.3. Canine-specific decidualization

As already indicated, despite strong exposure of the canine uterus to P4 before implantation, decidualization in the dog is not spontaneous. The first functional and biochemical changes in the uterus are evoked by contact with free-floating embryos [9,12,39]. Some of the factors more abundantly expressed in the uterus, e.g., PRLR, PTGES, IGFs, indicate an early ongoing decidualization. The first morphological changes are associated with embryo attachment as shown in investigations with early pregnant uteri at day 17 of pregnancy (embryonal life) [9]. Thus, together with the strong endometrial proliferation, formation of subepithelial, vimentin (mesenchymal cell marker) positive, enlarged, rounded cells is observed, indicating the ongoing decidualization process of stromal compartments [9]. These rounded cells with dense chromatin and ovoid nuclei were previously described as NEP/CD10-negative cells characteristic of the early pregnant canine uterus, resembling predecidual cells of women [122].

From then on, following implantation and placentation the contact between the trophoblast and uterine structures becomes more intimate on the way to formation of the endotheliochorial girdle placenta [36,37,123]. A strong morpho-functional rearrangement of the endometrium takes place, involving modification of ECM and remodeling of major collagens (COL1, -3 and -4) [40]. As a part of this process, highly specialized canine-specific maternal decidual cells develop. Together with maternal endothelial cells, these cells can restrain the proteolytic activity of invading trophoblast. In the fully developed placental labyrinth, decidual cells can be found in close contact with maternal blood vessels, and can be identified by smooth muscle α-actin and vimentin staining (both mesenchymal cell markers) [9,10]. In contrast to other species, PRL is not expressed in the canine uterus and placenta. However, the involvement of PRL in placentation and decidualization appears plausible. The respective receptor, PRLR, is abundantly present in the pre-implantation canine uterus, predominantly in

epithelial compartments, as well as in the invasive trophoblast following placentation [80], and circulating levels of hypophyseal PRL increase during placentation.

Also, prostaglandins, in particular PGE2, appear to be important candidates contributing to the process of decidualization in the dog, as shown similarly in human and rat decidua [121,124]. Besides being an important luteotropic factor in the dog, the respective synthase (PTGES) is concomitantly abundantly expressed in hatched pre-implantation embryos and in the early pregnant uterus [39]. Following placentation, PTGES and both of its cAMP-mediating receptors (PTGER2/-4) are strongly represented in fetal trophoblast [20].

As a functional characteristic, decidual cells represent the only population of canine placental cells expressing the nuclear P4 receptor (PGR) [21,125]. Any interference with their functionality at the level of PGR, e.g., by applying an antigestagen, will unequivocally lead to pregnancy loss (abortion/pre-term parturition) [21,41]. The same signaling cascade appears to apply in the normal prepartum luteolysis (reviewed in Refs. [5,7]). Another feature of canine decidual cells involves their expression of ERα and oxytocin receptor (OXTR) [125,126]. In particular, the presence of OXTR appears to be of functional importance as it could be involved in the luteolytic cascade [126].

3.4. An in vitro model of canine decidualization

An *in vitro* protocol has been developed with naturally estrogenized canine uterine stromal cells isolated during early diestrus [10]. The cAMP-mediated approach was applied and cells were morphologically and functionally characterized. Recently, we have further developed this model and established a new and unique cell line with immortalized dog uterine stromal (DUS) cells by stably transfecting them with the pSV40Tag oncogene [9]. Cells maintained their mesenchymal character and genomic incorporation of pSV40Tag for over 30 passages. When submitted to cAMP-mediated decidualization, they showed elevated levels of selected decidualization markers, e.g., PRLR, PTGES or IGF1 [9]. Furthermore, using DUS cells the basic decidual capability of PGE2 was validated. One of the most interesting findings from this study was the stimulatory effect of PGE2 upon PGR expression in decidualizing DUS cells, implying the involvement of PGE2 in PGR-mediated decidualization in the dog [9].

In our most recent, as yet unpublished studies, we found increased expression of COL4 in cAMP-decidualized DUS cells. This, taking into consideration the concomitantly maintained expression of vimentin, implies an epithelioid-like transition of canine decidual cells with still retained mesenchymal character, as described previously for humans [104].

Furthermore, our results indicate that, although PGE2 is involved in decidualization and regulation of expression of the respective markers, it does not participate in the regulation of extracellular matrix, at least concerning the expression of major collagens or ECM1 (*unpublished*).

4. Luteolytic cascade

Contrasting with non-pregnant dogs, around day 60 of pregnancy, the so far slowly progressing CL regression turns into a steep prepartum P4 decline, signaling the onset of parturition [24]. At this time, the progressively diminishing P4 levels seem to reach a lower threshold level and the prepartum luteolytic cascade becomes activated. The placental source of PGF2α appears to be in the fetal trophoblast which exhibits high expression of the required synthetic machinery (e.g., PTGS2/COX2) [21]. An alternative route of PGF2α synthesis was proposed, involving the conversion of PGE2 to PGF2α [20]. The increased expression of placental PTGES, accompanied by suppressed levels of PGE2 receptors, together with the respective biochemical activity of placental microsomal fraction at the time of increased PTGS2 availability, facilitate this process [20].

Due to their expression of PGR, maternal decidual cells play an important signaling role in the underlying feto-maternal communication, resulting in induction of prepartum luteolysis (reviewed in Refs. [5,7,8]). Alterations in this dialog by withdrawal of PGR activates the cascade. This is evident from experiments utilizing specific PGR blockers (antigestagens), resulting in a similar signaling cascade. The participation of OXTR is also implied, since its placental expression increases strongly both during normal and induced luteolysis [126], and it was also implied to participate in prostaglandin synthesis in other species [127]. Other than that, the expression of glucocorticoid receptor (GR/NR3C1), a mediator of cortisol effects localized in the fetal trophoblast, is strongly elevated during normal luteolysis, but remains unchanged when the prostaglandin cascade is induced by antigestagens in mid-gestation [28]. This interesting observation indicates that upon withdrawal of PGR activity, elevated levels of GR/NR3C1 are not needed to induce prepartum PGF2α release. Thus, the PGR signaling appears to be downstream of GR/NR3C1 in activating fetal prostaglandin synthesis. Notably, in human term placenta, a role for GR/NR3C1 as a local antigestagen in the luteolytic cascade taking place under otherwise high P4 concentrations was suggested [128]. As a prerequisite for such a mechanism, GR/NR3C1 was shown to bind P4 [129], whereas at physiological concentrations cortisol does not bind to PGR [130]. It remains to be verified whether a similar mechanism of local withdrawal of P4 due to increased availability of GR/NR3C1 (stimulated locally by fetal cortisol) applies to the dog. At least to some extent, this could explain the natural, but not antigestagen-induced, increase in GR/NR3C1 and the possible local effects of otherwise strongly varying cortisol levels [24,25,27]. However, the possible binding of aglepristone, as used in our studies, to GR/NR3C1 is not clear. Nevertheless, such a mechanism would involve competition between GR-expressing fetal trophoblast cells and maternal decidual cells for binding P4. There are no other antigestagen mechanisms known for the canine placenta that would regulate local P4 availability. Having observed variably low levels of circulating P4 in parturient bitches [17,27,31], the existence of a local antigestagen mechanism could be at least in part explanatory.

Recently, a deeper insight into molecular mechanisms of luteolysis was obtained by applying a transcriptomic approach to assess global placental changes associated with the prepartum luteolysis during normal parturition and abortion induced in mid-pregnant dogs with the antigestagen aglepristone [11]. Molecular patterns, gene networks and functional gene ontologies were validated, including those mediated through P4/PGR signaling. Cumulatively, the results obtained from this study [11] indicate that, besides inducing luteolytic and/or myocontractile output of placental PGF2α, both natural and induced withdrawal of P4 results in disruption of the feto-maternal interface, leading to alterations in vascular functions, apoptosis and controlled modulation of a pro-inflammatory immune response.

Among functional terms mapped by overrepresented genes during prepartum luteolysis when compared with mid-gestation, were those related to apoptosis, negative regulation of cell-matrix adhesion, negative regulation of endothelial cells and hypoxia, and increased cholesterol transport. This picture has been further completed by the main terms overrepresented during induced luteolysis, which besides relating to apoptosis, also included similar and related terms like those at normal luteolysis, e.g., negative regulation of endothelial cells, increased immune cell adhesion and diapedesis, leukocyte extravasation and suppression of cell cycle.

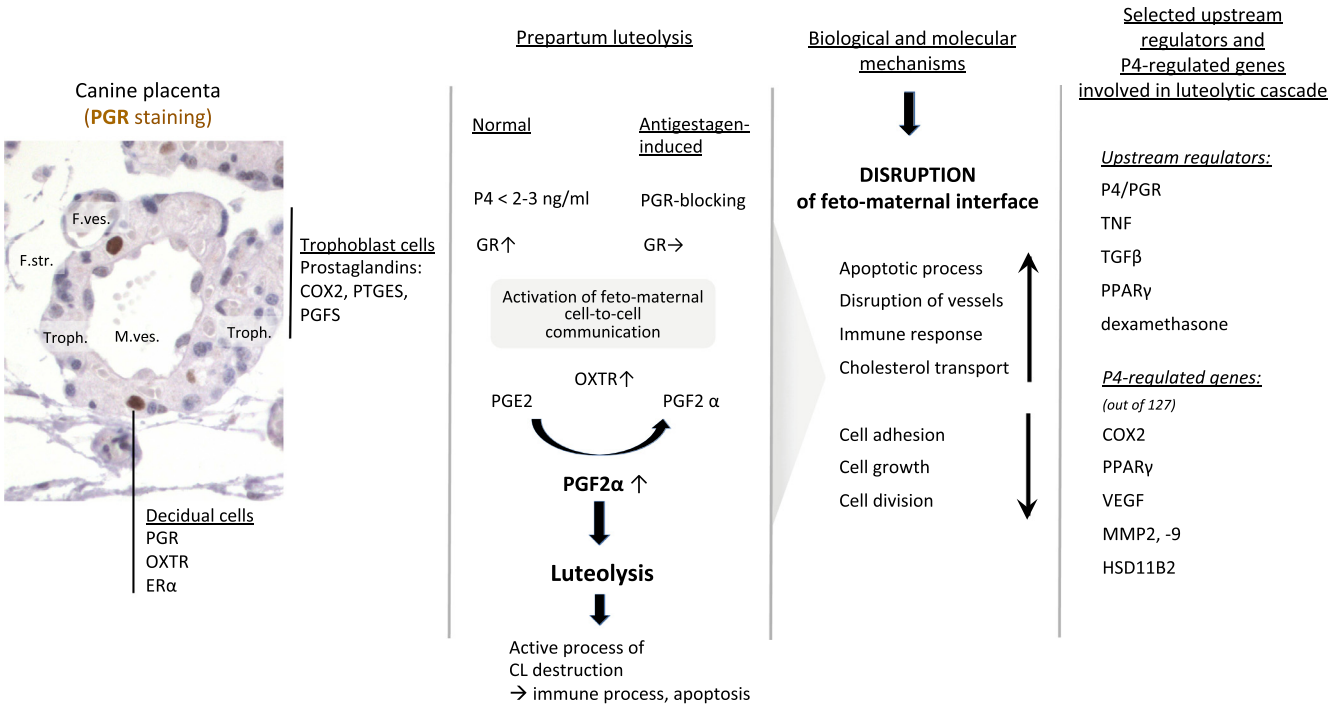

Fig. 1. Proposed model of luteolytic cascade (detailed explanations are provided in text).
PGR, nuclear progesterone (P4) receptor; OXTR, oxytocin receptor; ERα, estrogen receptor α; COX2 (PTGS2), cyclooxygenase 2; PTGES, prostaglandin (PG) E2 synthase; PGFS, PGF2α synthase (AKR1C3); GR (NR3C1), glucocorticoid receptor; TNF, tumor necrosis factor α; TGFβ, transforming growth factor β; PPARγ, peroxisome proliferator activated receptor γ; VEGF, vascular endothelial growth factor; MMP2/-9, matrix metalloproteinases 2/-9; HSD11B2, hydroxysteroid 11-beta dehydrogenase 2; CL, *corpus luteum*; M.ves., maternal vessel; F.ves., fetal vessel; F.str., fetal stroma; Troph., trophoblast (syncytio- and cytotrophoblast); ↑, increased; →, unchanged.

Among the top functional pathways were IL8-, IL3-, NF-κB- and TGFβ-signaling, but also those pathways related to vascular system, such as angiopoietin- and endothelin signaling [11]. Also, these pathways were shared between the two luteolytic groups. Indeed, many of these observations are consistent with some previous reports, such as strong apoptosis and detachment of the trophoblast reported for aglepristone-treated dogs [131], and corresponding with hypoxia and induced endothelin-1 signaling [132]. The induction of inflammatory reaction in the placenta upon withdrawal of P4/PGR signaling proves the anti-inflammatory properties of P4 also in the canine species, and is in accordance with data previously published by others [131,133].

Interestingly, although similar, all the respective functional changes were more pronounced during normal luteolysis. This indicates possible time-related effects associated with the placental maturation and/or priming of the placental tissue by decreasing P4 concentrations in the normal luteolytic group compared with the mid-pregnant aglepristone-treated group. Notable are the top upstream regulators found in both groups, including, i.a., P4/PGR, TNF, TGFβ, PPARγ, but also dexamethasone, the latter bringing us back to the above discussed, yet unresolved role of glucocorticoids during induction of parturition. We also found 127 P4-regulated genes that were differentially expressed during prepartum luteolysis. Some of these genes and upstream regulators include factors previously investigated and implicated in the luteolytic cascade, such as PTGS2/COX2, PPARγ, VEGF or MMPs, including MMP2 and MMP9 [21,134–136]. Some other genes included TGFβ family and IGF family members or HSD11B2. Several of the P4-dependent genes were shared between the two luteolytic groups.

Cumulatively, these findings and the comparison established between normal and induced luteolysis at the level of placental transcriptomes, support our previously postulated hypothesis of the importance of functional withdrawal of P4 for initiation of the prepartum luteolytic cascade in the dog [5,8,11,21]. However, as indicated above, direct comparison between the two groups revealed higher expression of involved genes and stronger manifestation of the respective functional pathways and networks during normal luteolysis, apparently leading to lower PGF2α output [21,137] or weaker uterine contractions observed mostly in induced abortions [138–140] and mirrored in different clinical outcomes.

Above, only some of the important facts and findings from the complex genomic analysis are highlighted. More comprehensive analysis of the data, including possible clinical implications, is presented in the source publication [11].

The proposed model of luteolytic cascade in the dog in presented in Fig. 1.

5. Final remarks

After having emphasized the origin and role of maternal decidual cells, it becomes clear that investigating the biology of the species-specific decidualization process holds one of the keys to understanding the regulation of establishment, maintenance and termination of canine pregnancy. Following this line of research, as presented herein, it is evident that the canine placenta is an important and highly sensitive endocrine organ. It provides a functional platform for embryo-maternal communication, which is essential for sustaining canine pregnancy and which in turn is dependent on P4 signaling.

Declaration of competing interest

Authors declare that there is no conflict of interest.

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